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14. ABSTRACT

Project rationale and original aims: New evidence suggests that Fragile X Mental Retardation Protein (FMRP) may need to associate with ancillary factors to co-regulate the production of some of important synaptic proteins. Currently, there is a critical need to identify and characterize these molecules. Some factors believed to associate with FMRP and co-regulate target mRNAs include a group of small regulatory molecules called microRNAs (miRNAs). There are two long-term goals associated with this project. First, to use an established model system for FXS to identify and functionally characterize all FMRP-associated miRNAs involved in the control of synapse structure and function. Second, to identify and functionally characterize all mRNA targets for these miRNAs. **Progress towards completion of aims:** First, after setbacks developing novel transgenic epitope-tagged FMRP protein that works efficiently in our assays, we have adapted two established antibody-based approaches to sequence FMRP-associated miRNAs and mRNAs: 1) direct co-immunoprecipitation of FMRP-containing RNPs (FMRP-IP); and 2) high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP). The latter will allow us to identify FMRP-associated mRNAs and specific FMRP binding sites. Second, a serendipitous discovery as allowed us to make substantial progress towards understanding the molecular mechanisms involved in FMRP-mediated recruitment of miRNAs to target mRNAs. This will allow us to very rapidly and efficiently validate bona fide target mRNAs.

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INTRODUCTION:

Fragile X Syndrome (FXS) is the most common form of inherited mental retardation in humans and affects an estimated 1 in 4000 males and 1 in 8000 females in the general population. FXS is characterized by an array of intellectual and emotional problems including learning disabilities, developmental delay, and anxiety. In the brain, the neuroanatomical defects associated with FXS include an abnormal synaptic morphology. This alteration in synaptic architecture is thought to be central to the pathology of FXS. At the genetic level, FXS is caused by a mutation in the Fragile X Mental Retardation gene 1 (FMR1). In normal cells, FMR1 produces a protein called the Fragile X Mental Retardation Protein (FMRP). The normal function of FMRP is to bind to messenger ribonucleic acids (mRNAs) and turn off the production of important proteins required in the control of synapse structure and function. Current hypotheses regarding the pathology of FXS have focused on this conserved function for FMRP. The **idea** is that in FXS, key mRNA targets of FMRP are dysregulated leading to the development of synaptic defects. Unfortunately, little is known about the specific mRNAs that are controlled by FMRP or exactly how those mRNAs are regulated. Until all of these processes have been fully characterized, further progress towards developing a treatment for FXS will be hindered.

New evidence suggests that FMRP may need to associate with ancillary factors to co-regulate the production of some of important synaptic proteins. Currently, there is a **critical need** to identify and characterize all of these molecules. Some factors that are believed to associate with FMRP and co-regulate target mRNAs include a group of small regulatory molecules called microRNAs (miRNAs). With this in mind, there are two **long-term goals** associated with this project. First, to use an established model system for FXS to identify and functionally characterize all FMRP-associated miRNAs involved in the control of synapse structure and function. Second, to identify and functionally characterize all mRNA targets for these miRNAs. These long-term goals are obviously well beyond the scope of this proposal and will be examined in future experiments. As such, one primary outcome of this project is **expected** to be the accumulation of preliminary data sufficient to obtain funding for this project at the NIH Research Project Grant (R01) or equivalent level.

This work is believed to be **significant** because it represents the first logical step in a series of experiments expected to lead to the discovery of new potential therapeutic targets for the diagnosis, prevention, and/or treatment of FXS. The basic **idea** behind the work proposed here is simple. First, all miRNAs that associate with the FMRP protein in neurons will be identified using **innovative** techniques. Second, candidate mRNA targets for each of these miRNAs will be identified by bioinformatics. The emphasis will be on mRNAs with a characterized role in the control of synapse structure or function. Finally, interactions between miRNAs and potential mRNA targets will be confirmed using established approaches. This work is **expected** to rapidly identify novel candidates that associate with FMRP. This alone would represent a significant advance in the FXS field. However, it will be necessary to demonstrate a mechanistic link between FMRP, FMRP-associated miRNAs, and target mRNAs in the pathology of FXS in future experiments.

BODY:

The following description of research accomplishments will be organized as outlined in the approved statement of work. Due to **unforeseeable complications**, we have been forced to deviate from this statement of work in several important ways (each will be indicated below). These deviations are primarily in methodology and in no way change the overall Aims of the project. That said, we had proposed a very tight timeline in the approved statement of work. We intend to request a **no-cost extension** towards the end of the funding period in order to complete the all proposed experiments.

Aim 1. Purification and deep sequencing of FMRP associated sRNAs (months 1-6).

Aim 1a_1. Purification of FMRP-associated sRNAs (months 1-2).

At the time the grant was submitted, we had developed but not tested transgenic fly lines that inducibly expressed a Protein A (PrA) tagged full length FMRP protein. The rationale for using PrA-tagged FMRP was primarily as a cost-saving measure [1]. These constructs would have allowed us to significantly scale-up our pull-down experiments with very little expense. We have since tested these constructs extensively *in vivo*. Unfortunately, these tagged constructs have not performed per our expectations. First, when expression of transgenic PrA-tagged FMRP was driven in the adult *Drosophila* brain, we did not observe an expected shift in size when Western blots were probed with an anti-FMRP antibody (**Figure 1A**). Second, when blots were probed with an IgG antibody (which should bind to the PrA tag), we could not see the PrA tag (**Figure 1C**). Finally, when PrA-tagged FMRP was immunoprecipitated from brain extract using IgG-conjugated magnetic beads (Dynabeads; see below), the FMRP protein was pulled down in both control and PrA-tagged FMRP expressing extracts (**Figure 1B**). While there was clearly more FMRP in the PrA-tagged FMRP expressing extracts, the additional protein runs appears to run at exactly the same size as endogenous FMRP. We believe that the PrA tag is likely being cleaved from FMRP during processing of the transgenic protein *in vivo*. **Based on these data, we decided to shift to an alternative strategy for the purification of FMRP.** It is important to note that each of these strategies must be done on a much smaller scale and require larger volumes of expensive antibody (anti-FMRP, -HA).

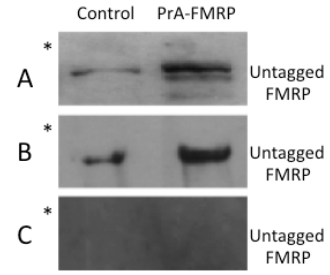

Figure 1. PrA-tagged FMRP. (A) Bands are the size of untagged FMRP. * indicates the expected size of PrA-FMRP (B) Endogenous FMRP is IP'd flies both the control and PrA-tagged FMRP flies. (C) The PrA tag is not recognized by specific antibodies.

Next, we developed a HA-FLAG-tagged FMRP protein. We initially **validated** the construct in *Drosophila* S2 cells to confirm that the tagged version of the protein was expressed (**Figure 2A**). Next, we confirmed that the HA-FLAG-tagged FMRP could be immunoprecipitated from S2 cell extract (**Figure 2B**). Unfortunately, we have not confirmed that the HA-FLAG-tagged FMRP protein can consistently co-immunoprecipiate proteins that are expected to interact directly with FMRP (**Figure 2C**) [2,3]. That said, we have developed a stably transfected cell line that could be useful for large-scale purification. We have also recently constructed a transgenic *Drosophila* line that constitutively expresses the HA-FLAG-tagged FMRP protein. This line is available but has not been tested further because of advantages of the third approach we are pursuing (see below).

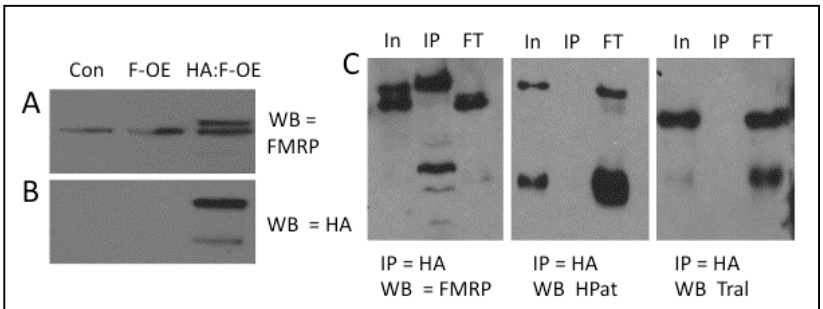

Figure 2. HA-FLAG-tagged FMRP. (A-B) Con = control; F-OE = untagged FMRP overexpression; HA:F-OE = HA:FLAG-tagged FMRP overexpression. Western blots against (A) FMRP and (B) the HA tag. (C) In = input; IP = immunoprecipitation; FT = flow-through. IP'd with antibodies against HA. Western blots against FMRP and the P body components, HPat and Tral. These should co-IP with FMRP [2, 3].

We next tried FMRP antibody covalently crosslinked to magnetic Dynabeads (Invitrogen). Dynabeads are very small and should provide a significant improvement in immunoprecipitation efficiency [1]. In our hands, crosslinked FMRP antibody did not always efficiently pull down FMRP from fly brain extract (as shown in **Figure 3A**) and inconsistently pulled down proteins we have previously shown to co-immunoprecipitate with FMRP such as Me31B and Tral (**Figure 3B-C**) [2]. This could be due to FMRP consistently crosslinking to Dynabeads in such a way that the epitopes that target FMRP are masked (Invitrogen).

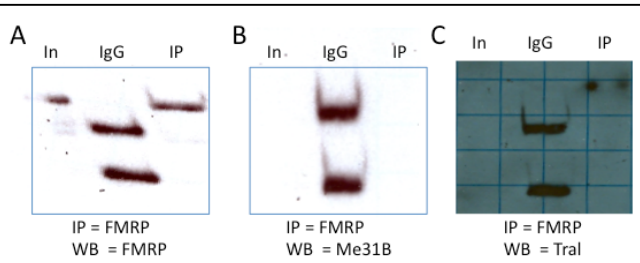

Figure 3. FMRP antibody covalently bound to Dynabeads. (A-C) In = input; IgG = IP with a normal mouse IgG; IP = IP with a FMRP antibody. Western blots against (A) FMRP, (B) Me31B, and (C) Tral. Again, these proteins have been shown to interact with FMRP [2].

Based on the complications described above, we have currently **deviated** from this approach in the following ways. First, we are using a proven (albeit more costly) approach to immunoprecipitate FMRP protein using FMRP antibodies that are non-covalently bound to commercially available Protein

A/G-conjugated magnetic Dynabeads (Invitrogen). This approach much more consistently pulls down FMRP as well as proteins that should co-immunoprecipate such as HPat (**Figure 4**). Second, to help prevent non-specific interactions can be problematic with Dynabeads, we are also using FMRP antibodies bound (non-covalently) to Protein A Sepharose [2,4]. This is a modification of a technique used to pull down FMRP-associated mRNAs [5]. Second, are using of a second approach recently used to identify FMRP binding sites in mammalian mRNAs [6]. The technique is called **HITS-CLIP** or high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation [7]. This approach will not only allow us to identify FMRP-associated mRNAs (i.e. Aim 1a_1) but also FMRP binding sites. We are currently moving forward with these approaches to independently purify mRNAs and miRNAs for sequencing.

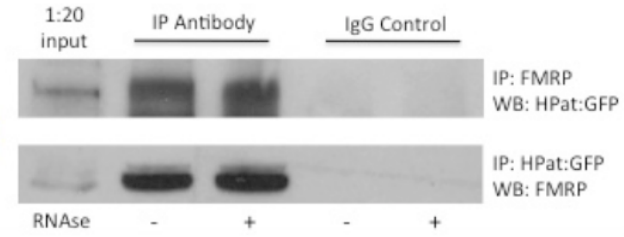

Figure 4. FMRP antibody pulled down with PrA/G Dynabeads. IPs from adult fly brain extract with antibodies against FMRP (top panel) or GFP (to pull down a HPat:GFP fusion protein; bottom panel). Western blots probed with either FMRP or GFP. These particular samples were also treated with RNAse as indicated.

Aim 1a_2. Deep sequencing of sRNA libraries and data analysis (months 3-6).

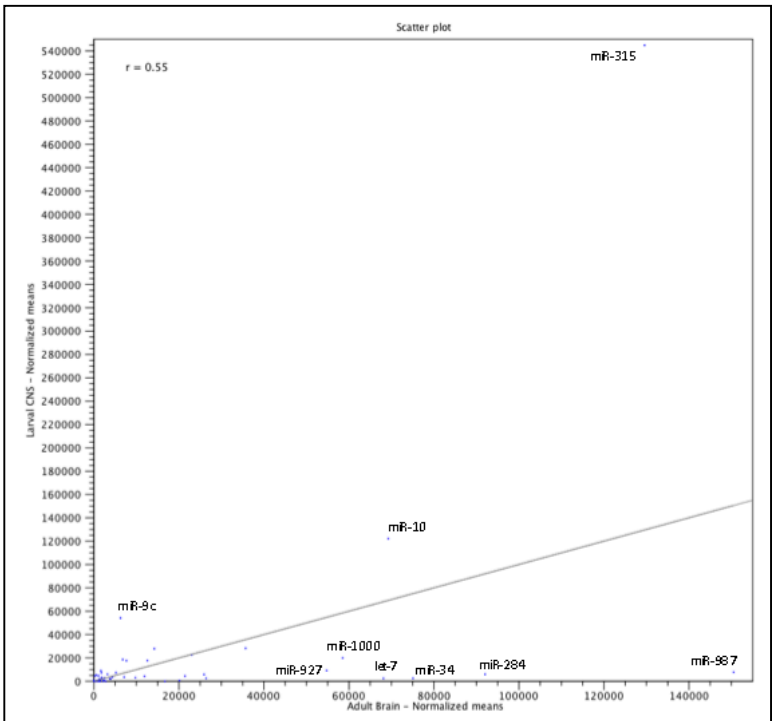

Figure 5. Relative expression of miRNAs in the adult brain compared to the larval CNS. Adult and larval brains share the expression of 147 miRNAs. Of those, three are enriched in the larval CNS (miRs-9c, -10, and -315). Six are enriched in the adult brain (miRs-927, -1000, -34, -284, -987, and let-7).

We anticipate completing sequencing (Aim 1a_2) within the **next several months**. The delay at this point is **expanding wild-type flies** sufficiently for large-scale immunoprecipitation. In the interim, we have identified an alternate vendor that can provide high quality RNA-seq data at a fraction of the cost. We will do our own data analysis using the CLC Genomics Workbench software package (CLC Bio). As proof of concept (and to establish a baseline for all miRNAs found in the *Drosophila* brain), we have done RNA-seq experiments to identify miRNAs found in explanted adult fly brains (**Table 1**). In summary, we identified a total of 169 miRNAs in the adult brain. 80 of these miRNAs met the cutoff outlined in our original grant proposal of 100 reads-per-million (RPM). This relative abundance data will be essential when assessing whether a particular FMRP-associated miRNA might have a biological function in the brain. For example, a miRNA that has a very low copy number may not be biologically relevant. We have also done comparative analysis to identify miRNAs that are enriched in the adult fly brain compared to the larval CNS (**Figure 5**).

We have also done a series of very targeted co-immunoprecipitation experiment to identify miRNAs that pull down with FMRP using anti-FMRP antibodies covalently linked to Dynabeads [4]. While all of these miRNAs (miRs-315, -9a, -9b, and -9c) are relatively abundant in the CNS (**Table 1**), none of the four miRNAs tested were enriched with FMRP when analyzed by RT-qPCR (**Figure 6)**. This could potentially be because crosslinked FMRP antibody does not efficiently pull down FMRP from fly brain extract (as explained in the text associated with **Figure 3**). Alternatively, these four miRNAs may not be associated with FMRP-containing complexes in fly neurons.

Milestone. Quality sRNA libraries have been generated and novel FMRP-associated miRNAs identified.

This milestone has not yet been reached for the reasons described above.

Aim 2. Identification and validation of target mRNAs (months 7-18).

Aim 2a. *In silico* identification of target mRNAs for all miRNAs identified in Aim 1a (months 7-9).

We have made a **serendipitous discovery** that will allow us to rapidly identify FMRP-associated mRNAs that are translationally repressed via the miRNA pathway. These studies will be discussed in more detail below. The bioinformatic analysis initially proposed in Aim 2 was **biased** to target mRNAs with **annotated functions** in the control of synapse structure or functions. In contrast, using results of these novel findings, our new focused approach will allow for the **unbiased identification of FMRP and miRNA-regulated mRNAs** and could potentially lead to **new gene discovery**.

Name	Reads	RPM
mir-184	212927	127871.87
mir-276b	202977	121896.47
mir-276a	201745	121156.6
mir-957	184563	110838.06
mir-14	133097	79930.505
bantam	86482	51936.181
mir-317	82009	49249.952
mir-999	65214	39163.828
mir-8	54554	32762.037
mir-277	40735	24463.129
mir-34	36987	22212.293
mir-987	31715	19046.229
mir-210	29284	17586.309
mir-315	27416	16464.494
mir-11	21702	13032.99
mir-133	21650	13001.761
mir-284	20288	12183.821
mir-10	17448	10478.279
let-7	15851	9519.2111
mir-981	15594	9364.8715
mir-927	12991	7801.6574
mir-1000	12326	7402.2961
mir-995	10293	6181.3917
mir-305	9982	5994.6227
mir-970	9870	5927.3619
mir-2b-2	7653	4595.9575
mir-278	7467	4484.2565
mir-2b-1	7331	4402.5826
mir-932	6060	3639.2921
mir-252	5512	3310.1944
mir-125	5078	3049.5586
mir-31a	4920	2954.6728
mir-990	4490	2696.4392
mir-1010	4010	2408.1784
mir-274	3531	2120.5182
mir-993	3256	1955.3688
mir-2a-2	3063	1839.464
mir-2a-1	2884	1731.9667
mir-263a	2880	1729.5646
mir-124	2670	1603.4505

Name	Reads	RPM
mir-33	2503	1503.1598
mir-279	2264	1359.6299
mir-13b-1	2097	1259.3392
mir-281-2	2092	1256.3365
mir-13b-2	2028	1217.9017
mir-190	1857	1115.2088
mir-306	1714	1029.3311
mir-9a	1664	999.30397
mir-7	1536	922.43443
mir-2c	1531	919.43172
mir-9c	1329	798.12198
mir-956	1181	709.24158
mir-1012	969	581.92641
mir-283	925	555.50251
mir-996	858	515.26611
mir-285	740	444.40201
mir-307a	736	441.99983
mir-1004	687	412.57321
mir-12	636	381.94551
mir-998	587	352.51889
mir-13a	583	350.11672
mir-929	568	341.10857
mir-137	507	304.47543
mir-1017	479	287.66022
mir-219	466	279.85316
mir-275	401	240.81784
mir-1003	350	210.19014
mir-100	340	204.18471
mir-2535b	319	191.5733
mir-92b	305	183.16569
mir-193	281	168.75265
mir-31b	272	163.34776
mir-9b	264	158.54342
mir-1001	263	157.94288
mir-375	253	151.93744
mir-281-1	238	142.92929
mir-1	235	141.12766
mir-971	223	133.92115
mir-1006	216	129.71734
mir-1009	179	107.49724

Table 1. RNA-seq analysis of miRNAs found in the adult *Drosophila* brain. The brain was explanted from young (~3 day old) adult wild-type flies *(*genotype is w^{1118} *Iso31*). Small RNA-seq was done at the University of Colorado Microarray Core facility (Aurora, Colorado). miRNAs are ranked in order of abundance in the adult fly brain. Reads (or tags) indicate the number of sequence reads that mapped to any variant of the indicated miRNA. Reads were then normalized to reads-per-million (RPM). As outlined in the original grant proposal, miRNAs with an abundance of less than 100 RPM are excluded from this table. The table shows the 80 most abundant miRNAs (out of 169 mapped reads in this tissue).

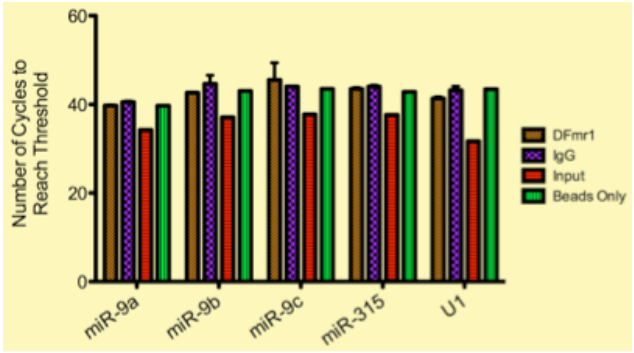

Figure 6. Targeted co-immunoprecipitation of FMRP associated mRNAs. The mammalian ortholog of miR-9 has been shown to co-IP with FMRP [4]. FMRP-containing complexes were IP'd from adult fly brain using FMRP antibodies covalently crosslinked to magnetic Dynabeads. dFMR1 = FMRP crosslinked beads. IgG = normal mouse IgG crosslinked beads. The U1 snRNA was used as a negative control. Note that no miRNAs are enriched with FMRP.

We have developed a novel *in vitro* reporter assay that allowed us to screen for genetic modifiers of FMRP-mediated translation repression. These experiments were based on a previously described tethering assay [8]. Briefly, *Drosophila* FMRP was fused to the λN protein that binds with high affinity to five BoxB stem loop sites (5xBoxB) cloned into the 3' UTR of a firefly luciferase reporter mRNA (FLuc-5xBoxB; **Figure 7A**). *Drosophila* S2 cells transiently transfected with the FLuc-5xBoxB reporter, a plasmid expressing λN-FMRP (or the λN peptide control), and a plasmid expressing *Renilla* luciferase (RLuc) as a transfection control. We observed a > 3-fold decrease in FLuc activity when λN-FMRP was expressed relative to the λN peptide control (**Figure 7B**). Surprisingly, this repression was almost completely eliminated when GW182, a core component of the miRNA pathway, is depleted from transfected cells by RNAi (**Figure 7B)**. Bearing this in mind, **GW182 RNAi** will be used to rapidly determine if a target of FMRP-mediated repression is co-repressed by miRNAs.

As an additional control for the λN-FMRP tethering assay, we asked if FMRP could repress FLuc-5xBoxB reporter activity in the absence of the λN protein tag. Surprisingly, we found that untethered FMRP was equally capable of repressing FLuc-5xBoxB reporter expression (**Figure 8A)**. We next asked what was the minimal number of BoxB binding sites required for FMRP-mediated reporter repression. We found that one BoxB site was sufficient to confer repression; 40% of control expression vs. 30% with the FLuc-5xBoxB reporter; p = 0.75). FMRP was capable of repressing activity of the FLuc-1xBoxB reporter in a concentration dependent manner (**Figure 8A**). In contrast, when the BoxB sites were removed, the ability to repress was eliminated. Together, these data suggest that FMRP can bind directly to one copy of a **stable stem loop**. We predict this represents a **novel FMRP binding site** in target mRNAs.

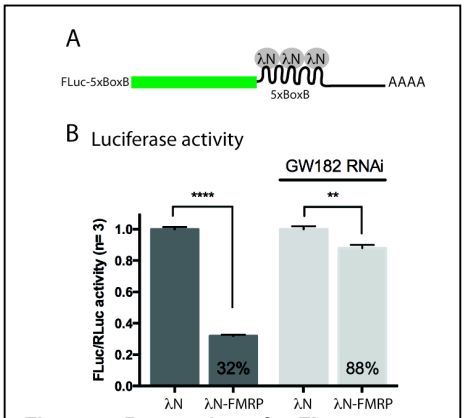

Figure 7. Repression of a FLuc reporter by tethered FMRP. (A) Structure of the FLuc reporter showing 5x BoxB sequences. (B) When FMRP is tethered to this reporter, it is capable of repressing translation (32% of control). This repression is reduced when expression of GW182 is inhibited by RNAi. ** p < 0.01; **** p < 0.0001. This suggests repression requires the miRNA pathway.

Why is GW182, an evolutionarily conserved effector of miRNA-mediated target silencing, required to regulate FMRP-mediated repression of the FLuc-5xBoxB reporter? It is possible that GW182 has a novel function in the control of general mRNA translation. That said, we instead hypothesized that it was more likely that FMRP was somehow repressing reporter expression via the miRNA pathway. The 3' UTR used in the FLuc-5xBoxB reporter is from the Simian virus 40 (SV40) small t antigen. We ran the SV40 3'UTR through the PITA algorithm against all known *Drosophila* miRNAs (miRBase v20). PITA identifies miRNA/mRNA target interactions based on both seed paring and thermodynamics [9]. Among these predicted miRNAs we identified miR-958-5p as a candidate (**Figure 9A)**. We were interested in miR-958 because: 1) **its predicted binding site was very close (~50 nt**) to the 5xBoxB sequence; 2) the predicted interaction energy was relatively low (ΔΔG = -12.27; the fourth strongest predicted interaction); and 3) we had already identified miR-958 as an activity-regulated miRNA required for activity-dependent axon terminal growth at the *Drosophila* NMJ [10]. First, we asked if miR-958 was capable of repressing the FLuc-5xBoxB reporter (**Figure 9B**). When we co-transfected S2 cells with a plasmid expressing the miR-958 primary transcript (pri-miRNA), we observed a > 3-fold decrease in FLuc activity relative to an empty vector control (p < 0.01). As we had predicted, this repression was completely eliminated when FMRP was depleted from transfected cells by RNAi (FMRP is highly expressed in S2 cells). Finally, we asked if FMRP binding to the BoxB stem loop is required to facilitate this repression. We found that miR-958 was incapable of repressing the activity of a reporter lacking the BoxB site. Together, these data strongly suggest that binding of endogenous FMRP to the **stable stem loop** is required for miR-958-mediated repression. The **proximity** of the miRNA and FMRP binding sites could potentially be used to quickly screen candidate mRNAs for further analysis.

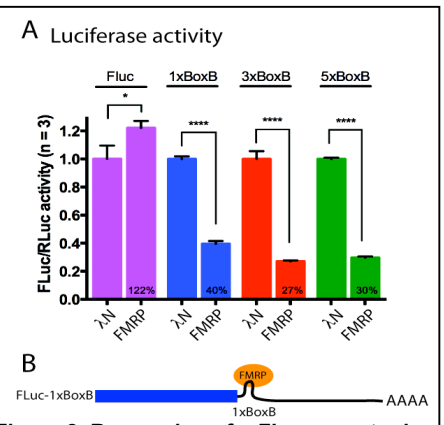

Figure 8. Repression of a FLuc reporter by untethered FMRP. (A) Untethered FMRP is capable of repressing 1x, 3x, and 5x BoxB-containing reporters. FLuc contains no BoxB stem loop sequences. (B) Structure of the 1xBoxB reporter. * p < 0.05; **** p < 0.0001.

As **proof of concept**, we identified known mRNA targets of FMRP that: 1) encode for proteins involved in the control of synapse structure or function; and 2) have been shown to interact directly with FMRP. Pickpocket (PPK1) is a

member of the DEG/EnaC superfamily of proteins and functions as a conserved Na^{2+} channel subunit [11]. *Drosophila* FMRP can **directly bind** to and downregulate levels of the *PPK1* mRNA *in vivo* [12]. This work also suggested that reduction of *PPK1* mRNA levels involved Ago2 (and potentially Ago1), the effector in the siRNA-induced silencing complex (siRISC). Thus, we asked if untethered FMRP was capable of repressing the activity of a FLuc-ppk 3'UTR reporter (**Figure 10)**. As observed with the FLuc-1xBoxB reporter, we found that increasing concentrations of co-transfected FMRP plasmid reduced FLuc-ppk 3'UTR reporter activity in a concentration-dependent manner. Interestingly, the *PPK1* 3'UTR is predicted to form **two highly stable stem loops** that are very similar to the BoxB site. We are in the process of confirming that FMRP targets the *PPK1* mRNA for miRNA-mediated translational repression and/or decay.

Aim 2b. Validation of miRNA/mRNA interactions for candidates identified in Aim 2a (months 10-18).

This Aim requires completion of sequencing in Aim 1a_2 and *in silico* analysis in Aim 2a. That said, Gateway cloning vectors (Invitrogen) have already been developed and the assays are already established in our lab [10].

Milestone. *Bona fide* mRNA targets for regulation by FMRP-associated miRNAs have been identified.

This milestone has not yet been reached.

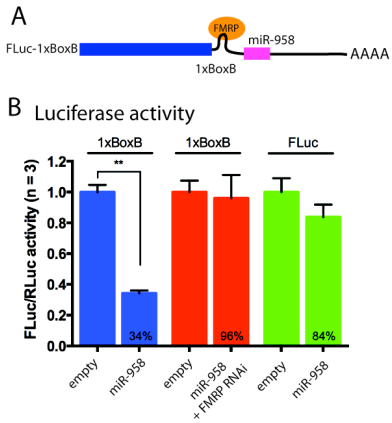

Figure 9. miRNA-mediated repression of the FLuc reporter requires binding of FMRP. FMRP is endogenously expressed in S2 cells. (A) Structure of the 1xBoxB reporter indicating the proximity of the miR-958 binding site. (B) co-transfected miR-958 is capable of repressing the 1xBoxB reporter. This repression is eliminated when FMRP expression is knocked down by RNAi or when the FMRP binding site is eliminated (in the FLuc reporter). ** $p < 0.01$.

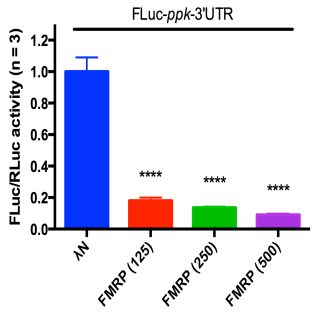

Figure 10. Dose-dependent repression of a FLuc-ppk-3'UTR reporter by FMRP. The ppk 3' UTR contains two predicted stem loops that are structurally similar to the BoxB sequence. FMRP is capable of significantly repressing translation of the FLuc reporter in a dose-dependent manner. FMRP (125) = 125 ng of transfected FMRP expressing plasmid. This is the same for all others. **** $p < 0.0001$.

KEY ACCOMPLISHMENTS:

1. An efficient small-scale FMRP immunopurification technique for miRNA purification has been developed.
2. An efficient small-scale HITS-CLIP technique for mRNA purification has been developed.
3. A rapid assay to confirm FMRP targets are co-regulated by the miRNA pathway has been developed.
4. A structural motif required for FMRP binding and recruitment of the miRISC has been identified.
5. A proximity requirement for FMRP and miRNA binding sites (~100 nt) has been identified.
6. The structural motif (stable stem loops) has been partially validated in a fly mRNA (3'UTR) that has been shown to directly interact with FMRP *in vivo*. This 3'UTR does not contain any other known structural motifs.

SUMMARY OF IMPORTANCE OF CURRENT FINDINGS:

- **Together, 1 and 2 will allow for the successful completion of Aim 1 within the next 3 to 6 months.**
- **Together, 3, 4, and 5 will allow us to optimize and improve the bioinformatic analysis outlined in Aim 2.**

REPORTABLE OUTCOMES:

Nothing to report at this time. The work is not yet completed.

CONCLUSIONS:

Nothing to report at this time. The work is not yet completed.

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